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13. ABSTRACT *(Maximum 200 Words)*

Epidemiological studies have consistently shown an early full-term pregnancy is protective against breast cancer. We hypothesize that the hormonal milieu that is present during pregnancy results in persistent changes in the pattern of gene expression in the mammary gland leading to permanent changes in cell fate that determine the subsequent proliferative response of the gland. To investigate this hypothesis, we have used suppression subtractive hybridization (SSH) to identify genes that are persistently up-regulated in the glands of estrogen and progesterone-treated Wistar-Furth rats 28 days subsequent to steroid hormone treatment compared to age-matched virgins. Using this approach, a number of genes displaying persistent altered expression in response to prior treatment with estrogen and progesterone were identified. Two markers have been characterized in greater detail - RbAp46, and a novel gene that specifies a non-coding RNA (designated GB7). Both were persistently up-regulated in the lobules of the regressed gland and required prior treatment with both estrogen and progesterone for maximal persistent expression. RbAp46 has been implicated in a number of complexes involving chromatin remodeling - thus suggesting a mechanism whereby epigenetic factors responsible for persistent changes in gene expression may be related to the determination of cell fate. These results provide the first support on the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland and can affect the response of mammary epithelial cells to carcinogenic insults. In addition, we have developed methods for culturing mammary epithelial cells from the rat and transducing them with a retrovial construct expressing a gene of interest (e.g. RbAp46). This approach will enable us to examine the role of a single molecule (such as RbAp46), in conferring protection against carcinogenesis, in the absence of the altered hormonal status that is present during pregnancy and without germline manipulation of the host animal.

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Introduction

Despite advances in the technologies for the diagnosis and treatment of breast cancer, strategies for the prevention of this disease are poorly defined. However, one fundamental concept arising from recent studies of mammary gland biology is the understanding that the processes involved in normal mammary development and carcinogenesis are intrinsically related (1). One of the most frequently cited examples of this principle is the protective effect of an early full-term pregnancy against breast cancer. There is strong epidemiological evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer (2, 3). This is substantiated by studies in a rat model in which prior treatment with estrogen and progesterone, (to simulate the effects of pregnancy) confers resistance to tumorigenesis in animals subsequently challenged with chemical carcinogen (4-12). The E+P-treated rat is thus a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. In this respect, the elucidation of novel targets for estrogen and progesterone action in the mammary gland, is crucial to our understanding of how an aspect of normal development might mediate the response of the organ to future proliferative signals.

To address this question, we have used the well-characterized Wistar-Furth rat model (described previously, 4), in conjunction with subtractive suppressive hybridization (SSH) methodologies (13) to identify markers that are persistently up-regulated in response to prior exposure to E and P in the mammary gland. As a result of these initial studies, we selected several specific markers to study in greater detail to investigate their role in relation to parity-related protection. To achieve this goal, we proposed to use mRNA expression analysis to study the temporal and spatial expression of each candidate biomarker, to evaluate their role as a function of reproductive history in relation to both differentiation and parity-related protection of the mammay gland. Furthermore, since the role of many of these markers has not yet been described in the mammary gland, we described experiments to further elucidate their function in relation to parity-related changes within the gland. In part, this approach will involve the use of novel retroviral transduction methods to overexpress the gene of interest in a parity-independent manner in the reconstituted mammary glands of recipient animals. If this approach is succesful,

then we have proposed experiments to investigate the influence of overexpression of a gene of interest in relation to protection against tumorigenesis following carcinogen challenge.

Report Body

a) Summary of research objectives

The specific objectives of this project, described in the original proposal are listed below:

Objective 1: To characterize the role of candidate biomarkers and to investigate their expression in the rat mammary gland:
a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, *per se* or of protection.

Objective 2: To investigate the function of candidate genes *in vivo:*
a) To investigate the function of GB7 and to determine its role during normal cellular processes
b) To investigate the function of GB7, RbAp and Nap1 in reconstituted mammary glands
c) To determine the role of systemic factors on parity-induced changes in the mammary gland.

The experiments described in objective 1 of the proposal are in progress and have already led to the preparation, and submission of a manuscript for publication. However, because of technical difficulties encountered in the first part of this objective, we have had to reorder the original statement of work to allow us to implement an alternative procedure to perform some of the analyses. This is described in greater detail in the Results section, below. In addition, because of the technical complexity of some of the experiments described in Objective 2, in particular those employing rat mammary epithelial cell culture and retroviral transduction methodology, we have already begun work on the experiments employing this technology. This has allowed us to take advantage of methods for the retroviral transduction of mouse mammary epithelial cells, established by two graduate students in Dr. Rosen's laboratory. Since one of these graduate students will depart shortly, to begin postdoctoral work in another laboratory, it is matter of expedience that we begin these experiments ahead of the original schedule to take advantage of his considerable technical expertise. This does not represent a significant departure from the original statement of work, however it has been necessary to reorder the time scale indicated in the proposal, such that objectives 1 and 2 are being investigated somewhat concurrently.

b) Results

Objective 1

a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, per se or of protection.

In the original statement of work, we proposed to use Northern analysis and *in situ* hybridization to investigate the expression of three markers (G.B7, RbAp46 and Nap1) at different stages of development in the rat mammary gland. In addition, we proposed to use perphenazine treatment as an experimental paradigm to distinguish the molecular processes involved in protection of the gland from those that are inherent to pregnancy-related differentiation of the gland. Because of the low abundance of these mRNAs, it was necessary to use poly A+ RNA to perform this analysis. One of the obstacles that we encountered was the technical difficulty of isolating sufficient amounts of poly A+ RNA from mammary glands at different stages of development to perform this analysis.

Because of these technical difficulties, we propose two alternative approaches to enable us to quantitate the expression of these mRNAs without the necessity of isolating poly A+ RNA. Firstly, Baylor has recently acquired the facilities for performing quantitative real-time RT-PCR. This will allow us to assess the levels of each mRNA in a total RNA sample isolated at different stages of development. This approach has the advantage of being extremely rapid and sensitive to small changes in the concentration of a reverse-transcriptase amplified cDNA product. This will circumvent some of the shortcomings associated with using poly A+ RNA Northern blots (such as the large amount starting material required to perform a few experiments and the long exposure time that is often necessary to detect hybridization signals). In addition, this should eliminate the necessity of pooling RNA samples from many animals, such that we can obtain a better measure of the variation in expression of each marker, by performing multiple replicates with samples from individual animals.

Secondly, we wish apply the use of cDNA microarray technology to quantitate the expression each marker at different stages of development. We have already begun experiments to investigate the effectiveness of this approach by constructing a pilot array, containing 96 non-redundant clones from the E+P subtracted SSH library (including G.B7, Nap1 and RbAp46) in conjunction with the Microarray Facility at Baylor College of Medicine. The cDNA inserts of

these clones were amplified using the nested primers in the SSH library backbone, purified by gel filtration chromatography and arrayed using silanized glass technology by means of the 384 well format array printer in the Microarray Core Facility. This approach has the advantage that it allows us to examine the expression of 96 markers in a single experiment, and to investigate changes in their expression, as a group, at different stages of development. From this analysis, it may be possible to predict markers that are involved in related pathways and this will be of great assistance to the functional studies described in Objective 2. Currently, we are optimizing the hybridization conditions for this technique, using the pilot E+P array described above. To this effect, we have investigated a different method of probe labeling that does not rely on direct incorporation of the Cy3- or Cy5-modified nucleotide. However, because the sensitivity of this method is still being evaluated, we propose to add a number of additional controls to examine the limits of detection of the array. To address this issue, we will prepare cDNA probes from known amounts of RNA derived from the *in vitro* transcription of an individual E+P clone and use this to screen the array. This will enable us to determine the lowest level of detection of the array and to predict its sensitivity with respect to low abundance clones. Secondly, we will screen the array with RNA isolated from the mammary glands of individual animals to obtain a measure of variability for the assay. Thirdly, to measure the linearity of the response we will compare the Cy5/Cy3 ratio from a hybridization containing the same probe labeled with both fluorescent nucleotides.

In the second part of this objective, we proposed to use an experimental paradigm based on previously published methods (11) to distinguish between the molecular processes involved in parity-related protection and differentiation of the mammary gland. This treatment results in an increased serum level of prolactin and progesterone, but not estrogen, which permits differentiation, but does not confer protection against carcinogenesis (11). To compare the morphological differentiation resulting from this treatment, parallel experiments were performed in which animals were treated with either E+P, blank pellets, perphenazine, or the vehicle alone for a period of three weeks. At the end of the treatment period, mammary glands were removed and the degree of morphological development examined by whole mount analsyis. Mammary glands from 6-, 12- and 18-day pregnant animals were used as positive controls for pregnancy-specific differentiation of the gland. At the gross structural level these glands appear to be very similar, although a few minor differences were observed. These observations are reported in

greater detail in our manuscript which is appended to this report (Appendix ii, Fig 1 and text). At the end of the 21 day-treatment period, hormonal stimuli were withdrawn and remaining animals in each cohort allowed to undergo involution for 28 days. This resting phase mimics the period of involution that occurs after a normal pregnancy and lactation and represents a period of extensive tissue remodeling. At the end of involution, the gland generally reaches a quiescent state, resembling that of the mature virgin animal. As reported in previous studies (4), similar morphological characteristics were observed in all of the treatment groups, indicating that there were no persistent structural differences as a consequence of hormonal stimulation of the gland. Please refer to the manuscript appended (Appendix ii, Fig 1 and text) for a detailed description of these observations.

In situ hybridization analysis was then used to examine the expression of two markers, G.B7 and RbAp46, in response to hormonal stimulation of the gland. The results of these experiments demonstrate that both markers are highly expressed during pregnancy. However, prior exposure to E+P (which simulates the protective effects of pregnancy) is required for maximal persistent expression of both genes 28 days following hormone withdrawal. Perphenazine alone (ie exposure to progesterone and prolactin) is not sufficient for persistent maximal expression of these genes (Fig 4 and 5 of manuscript, Appendix ii). These observations, and our interpretation of them, are discussed in greater detail in the appended manuscript (Appendix ii).

In the final part of this objective, we proposed to continue to characterize candidate markers by sequencing and Northern blot analysis. To address this goal, we have sequenced a total of 203 clones from the E+P SSH library (including those already presented in the original proposal). In addition, we have characterized 21 of these clones by Northern blot analysis, 18 of which were confirmed as differentially expressed, based on quantitative Northern analysis of their expression in the E+P-treated gland vs. AMV. The results of this analysis are presented in greater detail in Tables I-III and Fig 3 of the manuscript (Appendix ii).

Objective 2

a) To investigate the function of GB7 and to determine its role during normal cellular processes.

In the first part of this objective, we proposed experiments to determine the function of GB7 and to predict its role in normal cellular processes. Clone GB7 is a novel cDNA whose pattern of

expression suggests it is an excellent marker for parity-induced protection. However, preliminary database searches using in the ~750 bp of sequence obtained from the SSH library clone revealed no homology to any known cDNA or protein. Therefore, to further elucidate the function of this cDNA, we constructed and screened an E+P-treated mammary gland cDNA library and isolated several full-length clones corresponding to the different sized transcripts observed by Northern analysis (refer to Fig. 3 of manuscript, Appendix ii). Three of these clones (6.3, 2.4 and 2.2 kb in size) were sequenced in their entirety. Analysis of the resultant sequences suggested that the different-sized transcripts observed by Northern blot analysis arose as a result of differential splicing of a single gene product. The full-length sequence of the largest of these clones (6.3 kb in length) was deposited in Genbank, with the accession # AY035343.

In an attempt to determine the function of this gene, we submitted the full-length, 6.3 kb sequence to database searching using BLAST and TBLASTN search algorithms (with six-frame translation and BEAUTY post-processing). The results of this analysis did not reveal significant homology to any known gene or protein motif. However, we found that a short section of the 5' and 3' region of this gene (n.t. 1125-1490 and 5920-6203 of the full-length sequence) exhibited homology with several rat and mouse EST clones. The best matches were to accession #s BG079981, BE119249 and BG079981 and revealed an identity of 85-97 % to these regions of ~300 bp in the full-length G.B7 sequence. In addition, we performed homology searching using the recently assembled human genome sequence database and found four regions of homology to human chromosome 2. These homologous regions corresponded to n.t. 2334-2419, 2435-2601, 2745-2789 and 5157-5437 of the full-length G.B7 sequence, with identities of 93, 83, 95 and 80 % respectively ($E < 1^{-9}$). This sequence maps to region 2q33 of the human genome and spans a known chromosomal break-point, which is associated with a number of human cancers, including breast adenocarcinoma (14). No open reading frames (ORF) or ESTs have been identified in the human sequence encompassing the region of homology with G.B7. However this may be a consequence of incomplete annotation of genome database or the failure of the search paradigms to detect a non-translated RNA. Indeed, we have been unable to detect any significant ORFs longer than ~200 n.t. in the full-length G.B7 cDNA. *In vitro* translation experiments, with appropriate positive controls run in parallel to validate the assay, also failed to detect any translation product for either the 6.3 kb, 2.4 kb or 2.2 kb forms of this RNA.

b) To investigate the function of GB7, RbAp and Nap1 in reconstituted mammary glands

The second part of this objective proposed the use of reconstituted mammary gland experiments to elucidate the function of GB7, RbAp and Nap1 in an *in vivo* context. To determine the role of a single gene in conferring parity-specific protection, it would be advantageous to develop a system that would permit the over-expression of the molecule of interest in the absence of the altered hormonal status that is present during pregnancy. To achieve this goal, we have recently developed methods for growing rat mammary epithelial cells (MEC) in primary culture based upon technology for isolating epithelial cells from mouse mammary glands (which is now well established in our laboratory). Our approach is to use an HIV-derived lentivirus vector (15) pseudotyped with the vesicular stomatitis virus (VSV) G protein to enhance the efficiency and host range of infection. Lentivirus has advantages over many conventional retroviral strategies, such as the ability to transduce slowly- or non-dividing cells – features which are of particular importance for conferring the ability to transduce a population of stem cells (16). However, although this retroviral vector has been used to transform intact tissues (16), it has not been applied to the infection of MECs from rats. As a preliminary measure to examine the efficacy of this approach for infecting MECs from rats, we will use a control retrovirus expressing the bacterial *lacZ* gene to determine the efficiency of infection and viability of the isolated rat MECs. Once we have established the success of this strategy, we will develop a retroviral construct expressing a gene of interest (ie RbAp46 or G.B7) and use this construct to infect rat MEC, to determine the effects of overexpression of that gene on normal mammary gland development and in repsonse to carcinogen challenge.

Key Research Accomplishments

- Experiments involving perphenazine treatment have been reporduced in Wistar-Furth rats. Level of morphological development examined by whole mount analysis, in comparison to mammary glands for E+P-treated, pregnant and unstimulated control animals. (Objective 1)

- In situ hybridization analysis used to characterize the expression of G.B7 and RbAp46 in response to pregnanct, hormonal stimulation and perpehnazine treatment. (Objective 1).

- Screening of rat E+P SSH library complete. Twenty-one markers from this library characterized by Northern blot analysis to verfiy their pattern of expression. (Objective 1). Reverse subtracted library constructed and partially screened.

- Manuscript prepared and submitted for review

- Isolation and sequencing of full-length clones for G.B7 completed. Sequence sumbitted to Genbank (accession # AY035343). (Objective 2).

- Established methods for culturing mammary epithelial cells (MEC) from rats. (Objective 2)

Reportable Outcomes

Manuscripts:

Persistent Changes in Gene Expression Induced by Estrogen and Progesterone in the Rat Mammary Gland. Melanie R. Ginger, Maria F Gonzalez-Rimbau, Jason P. Gay and Jeffrey M. Rosen. Submitted to Molecular Endocrinology, 24 May, 2001. (Refer to Appendix ii)

Abstracts:

- AACR Meeting, San Francisco, April, 2000

- International Congress of Endocrinology Meeting, Sydney, Australia, October, 2000

- Hormones and Cancer Meeting, Port Douglas, Australia, November 2000

(refer to Appendix i)

Presentations:

- AACR Meeting, San Francisco, April, 2000. Oral presentation

- International Congress of Endocrinology Meeting, Sydney, Australia, October, 2000. Oral presentation

• Hormones and Cancer Meeting, Port Douglas, Australia, November 2000. Poster.

Informatics:

• Full-length G.B7 sequence submitted to Genbank database, under the accession # AY035343.

• Results of the E+P SSH library screening experiments will shortly be made publicly available through a web site for Dr. Rosen's laboratory.

Funding applied for, based on work supported by this award:

We have used the work presented in this report to apply for a renewal of NIH grant CA 64255, which if awarded, would be used to supplement costs associated with this project.

Summary and Conclusions

The E+P-treated rat is a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. We have hypothesized that the normal hormonal milieu that is present during pregnancy results in persistent changes in the molecular pathways governing cell fate in a defined population of cells in the mammary gland. These changes accordingly dictate the type of response that is elicited by subsequent exposure to hormones or chemical carcinogens. A critical aspect in understanding these processes is the elucidation of target genes for E and P in the mammary gland. Such information is necessary for determining how the molecular pathways involved in normal mammary development and tumorigenesis converge with systemic hormones to mediate parity-specific protection.

Previous studies, utilizing a rational approach to study known targets associated with proliferation and differentiation in the mammary gland, have met with limited success in revealing the molecular pathways involved in conferring the refractory state. Therefore, we employed an alternative strategy to identify novel targets for E and P action in the rat mammary gland. Using this Wistar-Furth rat model, in conjunction with subtractive suppressive hybridization (SSH) methodologies, we have identified several markers that are persistently up-regulated in response to prior exposure to E and P in the mammary gland. The expression of two

of these markers, RbAp46 and a novel gene that specifies a noncoding RNA (G.B7) has been characterized further and the results of this work is described in the appended manuscript.

In addition, we are developing methods for culturing mammary epithelial cells from rats and transducing them with a lentivirus vector expressing a gene of interest. Transduced cells will then be transplanted into the cleared fat pads of virgin rats, to produce reconstituted mammary glands, over-expressing a gene of interest. These experiments are essential to advancing our understanding of the role of these markers in normal mammary gland development and in response to carcinogen challenge.

What are the implications of this work?

The observation that pregnancy confers protection against breast cancer has lead to the proposal that breast cancer may be prevented by pharmacologically mimicking the specific endocrine events at a defined window of development. However, the advancement of such technology is currently limited by our ignorance of the complex molecular and cellular events that are responsible for this developmentally specific protection. Another factor impeding the implementation of such strategies is the prolonged nature of any study that must rely on a defined clinical endpoint (such as tumorigenesis) as a measure of the efficacy of any chemopreventative treatment. In this respect, the identification of relevant intermediate biomarkers for a defined population of resistant and susceptible cells is of critical importance, and will greatly facilitate the short-term evaluation of any chemopreventative measures and could ultimately improve imaging techniques for assessing breast cancer risk in women.

The elucidation of markers that show persistent changes in gene expression in response to exposure to E and P is critical for understanding the molecular pathways that are altered in the parous gland and modulate the response of the gland to further proliferative stimuli. In this study, we have identified a number of such markers that warrant further study. <u>These results provide the first support on the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland and can affect the response of mammary epithelial cells to carcinogenic insults.</u>

In addition to the identification of sutiable markers for following these parity-specific changes in the mammary gland, it is critical to develop a method of overexpressing these genes

in the rat mammary gland to enable us the study the effects of their overexpression in a parity independent manner. To this end, we are developing a strategy for transducing mammmary epithelial cells from rats and producing reconstituted mammary glands expressing a gene of interest. This approach will enable us to examine the role of a single molecule (such as RbAp46), in conferring protection against carcinogenesis, in the absence of the altered hormonal status that is present during pregnancy and without germline manipulation of the host animal.

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Appendix i

Meeting Abstracts

1. AACR Meeting, San Francisco, April, 2000.

INTERMEDIATE MOLECULAR BIOMARKERS FOR THE PROTECTIVE EFFECT OF EARLY PREGNANCY AGAINST BREAST CANCER.

M.R.Ginger, J.P.Gay, M.F.Gonzalez-Rimbau, & J.M.Rosen.
Baylor College of Medicine, Houston, TX, 77030.

The objective of this study is to identify intermediate molecular biomarkers of parity-related protection against breast cancer. To achieve this goal, we have used a well-established rat model to identify genes whose expression is persistently altered in the mammary gland in response to prior exposure to estrogen and progesterone (E+P). Using subtractive suppressive hybridization based PCR, we have identified a number of genes that are up-regulated by E+P treatment. While some of these markers relate to known genes (e.g. Nap1 and CDC42) whose role in cell proliferation processes have been previously reported, others (such as α-casein) are markers of differentiated mammary function. In addition, this methodology has resulted in the isolation of a high percentage of ESTs (27%) and novel genes (8%) whose function in the mammary gland have yet to be defined. Several candidate markers (including one novel marker designated G.B7) have been characterized in greater detail. Northern analysis has shown that GB7 is upregulated 4-to 8-fold in the glands of hormone-treated rats c.f. age-matched virgin (AMV) controls. *In situ* hybridization experiments also suggest that this gene is highly expressed in the ductal mammary epithelial cells of pregnant and parous animals, a pattern of expression that makes it biologically relevant as a marker of parity-induced changes in the mammary gland.
Supported by NIH grant CA 64255.

2. International Congrees of Endocrinology Meeting, Sydeny, Australia, October 2000

Persistent Changes in Gene Expression Induced by Estrogen and Progesterone in the Mammary Gland

Ginger, M.R., Gay, J.P. Gonzalez-Rimbau, M.F. and Rosen, J.M.
Baylor College of Medicine, Houston, TX, 77030, U.S.A.

Epidemiological studies have consistently shown that an early full-term pregnancy is a significant determinant of breast cancer risk. This is substantiated by studies in a rat model in which prior treatment with estrogen and progesterone (E+P), confers resistance to chemical carcinogen-induced tumorigenesis. Thus, we hypothesize that in the normal hormonal milieu that is present during pregnancy, there are persistent changes in the mammary gland that confer this resistance to tumorigenesis. To investigate this hypothesis, we have used a well-defined rat model to identify genes that are up-regulated in the glands of parous (E+P) rats, compared with non-parous age-matched virgin (AMV) animals. By using a dual approach, combining SSH-PCR, high-throughput screening and cDNA microarray technology, we have identified a number of genes whose expression is altered in response to prior treatment with E+P. While some of these genes (e.g. Nap1 and CDC42) may play a role in the cell cycle progression and control of proliferation, others (such as α-casein) appear to be markers of mammary differentiation. To distinguish markers that are specifically related to parity, we have treated rats with perphenazine (an agent which increases serum prolactin and progesterone, but not estrogen and causes near-lactional differentiation without conferring protection) to eliminate markers that are induced by differentiation *per se*. According to these criteria, two clones have been selected for further analysis: RbAp46 and a novel clone (designated GB7). Both genes are upregulated in a developmentally-specific manner and require estrogen and progesterone for enhanced expression. *In situ* hybridisation experiments suggest that both markers are specifically expressed in mammary epithelial cells of the lobular alveolar units of pregnant and parous animals, a pattern of expression that makes them biologically relevant markers of parity-induced protection. Recent studies, showing that RbAp46 is a component of the histone deacetylase complex, suggest a model in which chromatin remodelling provides a mechanism for persistent changes in gene expression following parity. Supported by NIH grant CA 64255 and DAMD fellowship 17-00-0137.

3. Hormones and Cancer Meeting, Port Douglas, Australia, November 2000

Development of a reconstituted mammary gland model to study the effects of overexpression of RbAP46, a marker for parity-specific protection against tumorigenesis.
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There is strong epidemiological evidence that women who experience an early full-term pregnancy have as significantly reduced risk of developing breast cancer. In experimental studies, this hormone-dependent parity-related phenomenon of protection against breast cancer may be simulated by treating rats with a defined dose of estrogen and progesterone at a specific stage of development. Hence we propose the normal hormonal milieu that is present during pregnancy results in persistent changes in the mammary gland that confer resistance to tumorigenesis. The long term objective of these studies is to identify suitable intermediate molecular biomarkers for assessing risk and the efficacy of preventative treatments. To investigate this hypothesis, we have used a well-defined rat model to identify genes that are up-regulated in the glands of parous (E+P) rats, compared with non-parous age-matched virgin (AMV) animals. Preliminary studies using this model, have lead to the identification of a number of candidate markers for this hormone related protective effect. One such marker, the Retinoblastoma (Rb)-associated proteins, RbAp46, has been selected for further analysis based on promising early expression data. RbAp46, and the closely related molecule RbAp48 comprise part of a small gene family with homology to the yeast molecule MSI1 (a negative regulator of the Ras-cyclic AMP signaling pathway) (1, 2). Although originally isolated as protein components that bound to Rb by affinity- chromatography, subsequent studies suggests that these molecules might play a broader role in the regulation of such processes as cellular proliferation and differentiation. In addition, to its interactions with Rb, RbAp46 appears to be a downstream target of the Wilm's Tumor suppressor protein (WT1), and has also been shown to interact with the breast cancer tumor suppressor protein, BRCA1 (3,4). Furthermore RbAp46 and RbAp48 interact directly with acetylated histones (5) and are components a core complex of chromatin associated proteins contained within the Sin3A/HDAC and NuRD complexes (6). Together these studies imply a model in which RbAp46 through its actions as an adapter protein, might serve as a means of recruiting these chromosome remodeling activities, leading to persistent changes in gene expression in the glands of parous animals. Because of these qualities, we are pursuing experiments investigate the role of RbAp46 in the mammary gland during the processes involved in normal development and tumorigenesis. We have recently developed methods for growing rat mammary epithelial cells (MEC) in primary culture and transforming them with recombinant retrovirus vector expressing a gene of interest. Our approach is to use an HIV-derived lentivirus vector pseudotyped with the vesicular stomatitis virus (VSV) G protein to enhance the efficiency and host range of infection. Lentivirus has advantages over many conventional retroviral strategies, such as the ability to transduce non- or slowly dividing cells - features which are of particular importance for conferring the ability to transduce a population of stem cells. Using this approach we have been able to achieve transfection efficiencies that are five to ten-fold higher than those achieved with conventional retroviral vectors. Transduced MEC are then transplanted into the cleared fat pads of 21 day old syngeneic host rats to examine the effect of overexpression of a gene of interest (in this case RbAp46) in development in a reconstituted mammary gland. This approach will enable us to characterize the role of a single molecule (RbAp46) in the absence of the altered hormonal status that is present during pregnancy and without germline manipulation of the host animal. Supported by NIH grant CA 64255 and DAMD fellowship 17-00-0137.

Appendix ii

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Persistent Changes in Gene Expression Induced by Estrogen and Progesterone in the Rat Mammary Gland.

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Persistent Changes in Gene Expression Induced by Estrogen and Progesterone in the Rat Mammary Gland.

Running title: Gene expression in the parous mammary gland

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Abstract

Epidemiological studies have consistently shown an early full-term pregnancy is protective against breast cancer. We hypothesize that the hormonal milieu that is present during pregnancy results in persistent changes in the pattern of gene expression in the mammary gland leading to permanent changes in cell fate that determine the subsequent proliferative response of the gland. To investigate this hypothesis, we have used suppression subtractive hybridization (SSH) to identify genes that are persistently up-regulated in the glands of estrogen and progesterone-treated Wistar-Furth rats 28 days subsequent to steroid hormone treatment compared to age-matched virgins. Using this approach, a number of genes displaying persistent altered expression in response to prior treatment with estrogen and progesterone were identified. Two markers have been characterized in greater detail - RbAp46, and a novel gene that specifies a non-coding RNA (designated GB7). Both were persistently up-regulated in the lobules of the regressed gland and required prior treatment with both estrogen and progesterone for maximal persistent expression. RbAp46 has been implicated in a number of complexes involving chromatin remodeling - thus suggesting a mechanism whereby epigenetic factors responsible for persistent changes in gene expression may be related to the determination of cell fate. These results provide the first support on the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland and can affect the response of mammary epithelial cells to carcinogenic insults.

Introduction

Breast cancer is one of the most frequently diagnosed cancers affecting women in the Western world. Despite advances in the technologies for the diagnosis and treatment of breast cancer (1), our understanding of the etiological factors contributing to the development of this disease is limited (2). However, one fundamental concept arising from studies of mammary gland biology is the understanding that the endocrine processes involved in normal mammary development and carcinogenesis are intrinsically related (3). One of the most frequently cited examples of this principle is the role of reproductive history and breast cancer risk. There is significant evidence that the timing of normal developmental events like menarche, menopause and parity and the duration of lactation have a significant impact on an individual s susceptibility to breast cancer (4, 5). In particular, there is strong epidemiological evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer (5-7). This is substantiated by studies in several rodent models that demonstrate that early pregnancy confers resistance to tumorigenesis in animals treated with chemical carcinogens (8-11). These studies also revealed a strong age-related component to the timing of developmental events and exposure to carcinogen (12-14). Thus, the regulation of normal developmental events governing the timing and period of exposure to ovarian hormones appears to be a critical factor in determining the risk of developing breast cancer. Furthermore, there appears to be a functional interplay between the endocrine processes controlling mammary development and carcinogenesis.

The observation that the protective effect of an early full-term pregnancy can be accurately reproduced in rodents has lead to the development of defined animal models for

studying this parity-related phenomenon. Huggins *et al.* initially demonstrated that treatment with the ovarian hormones, estrogen (E) and progesterone (P), could inhibit tumorigenesis in rats following prior exposure to chemical carcinogen (15). Since then, numerous investigators have extended these observations to show that hormonal manipulation (by treatment with estrogen and progesterone or human chorionic gonadotrophin), either prior to, or immediately following carcinogen exposure, can inhibit carcinogenesis by inducing a refractory state. These studies have been performed in a variety of rat and mouse strains (11, 16-23). Taken together, these findings provide strong support for the utility of the rodent model to define the molecular mechanisms by which a defined hormonal regimen can mimic the protective effect of pregnancy.

Despite the wealth of literature supporting the role of endocrine-mediated processes in parity-related refractoriness, little is known of the molecular mechanisms governing pregnancy-specific developmental changes in the mammary gland. Mammary gland development is mediated by a complex interaction between systemic hormones and local growth factors (24), which is in turn modulated by the topography of the cells receiving the stimuli (2). E and P appear to be key players in these processes (25). However, although distinct morphogenic functions have been associated with these hormones, the molecular pathways that are elicited in response to the combined effect of E and P signaling remain to be elucidated fully (24). Furthermore, it seems likely that the signaling pathways induced by these hormones vary depending on the context of the population of cells receiving the stimuli. Therefore, we hypothesize that the normal hormonal milieu that is present during pregnancy results in persistent changes in the molecular pathways governing cell fate in a defined population of cells in the mammary gland. These changes accordingly dictate the type of response that is elicited by subsequent exposure to hormones or chemical carcinogens. A critical aspect in understanding

these processes is the elucidation of target genes for E and P in the mammary gland. Such information is necessary for determining how the molecular pathways involved in normal mammary development and tumorigenesis converge with systemic hormones to mediate parity-specific protection.

Previous studies, utilizing a rational approach to study known targets associated with proliferation and differentiation in the mammary gland, have met with limited success in revealing the molecular pathways involved in conferring the refractory state (11, 21). Therefore, we employed an alternative strategy to identify novel targets for E and P action in the rat mammary gland. Conditions for the hormonal regimen were based on previous studies, which defined the minimal dose of E and P required to induce a level of morphological differentiation equivalent to that observed during a full-term pregnancy (11). These studies have shown that prior treatment with E and P confers resistance to chemical carcinogen-induced tumorigenesis in Wistar-Furth rats in a reproducible and statistically significant manner. By employing an experimental paradigm involving the administration of E and P, we have been able to circumvent the difficulties that are likely to be encountered by attempting to induce synchronized pregnancies within an age-matched cohort of animals. The use of a model employing just two hormones avoids the complex hormonal interactions present in a full pregnancy (11). Furthermore, this approach is likely to give a clearer picture of the molecular pathways that are influenced by estrogen and progesterone during normal development and tumorigenesis. The forty-five day old rat was used as model for the initiation of hormonal treatment because it represents a stage of development that is analogous to humans both in the rapid development of the mammary epithelium during puberty and the maximum susceptibility to carcinogenesis (25). Using this Wistar-Furth rat model, in conjunction with subtractive suppressive hybridization

(SSH) methodologies, we have identified several markers that are persistently up-regulated in response to prior exposure to E and P in the mammary gland. The expression of two of these markers, RbAp46 and a novel gene that specifies a noncoding RNA (G.B7) has been characterized in further detail.

Results

Morphological effects of hormonal treatments

To simulate the protective effect of a full-term pregnancy, 45-day old Wistar-Furth rats were treated with a defined dose of E and P as described in the Materials and Methods (Fig. 1A). In the expression studies presented below, we used perphenazine (PPZ) treatment to distinguish between the processes involved in differentiation and protection due to E + P or pregnancy. Using the experimental paradigm described previously (22), 45-day old Wistar-Furth rats were treated with PPZ for a period of three weeks. This treatment results in an increased serum level of prolactin and progesterone, but not estrogen, which permits differentiation, but does not confer protection against carcinogenesis (22). Hormonal stimulation (by either E+P or PPZ treatment) was verified by whole mount analysis of mammary glands at the conclusion of the 21-day treatment period. In both cases, mammary glands from 6-, 12- and 18-day pregnant animals were used as positive controls for pregnancy-specific differentiation of the gland. Figure 1B shows representative whole mount analyses of mammary glands from a 12-day pregnant, 21-day E+P- and PPZ-treated rats, and AMV rats. In the E+P- and PPZ-treated gland (Fig. 1 B, panels i and ii) we observed a level of differentiation that was comparable to that of a 12-day pregnant animal (Fig. 1B, panel iii). At the gross structural level these glands appear to be very similar, although a few minor differences were observed. In particular, the E+P-treated mammary gland (Fig 1B, panel i) appeared to exhibit a slightly greater degree of secondary and tertiary branching than the PPZ-treated animals (Fig. 1B, panel b), but slightly reduced lobuloalveolar development compared to the mammary glands isolated at day 12 of pregnancy (Fig. 1B, panel iii) or from the PPZ-treated animal (Fig. 1B, panel ii). The mammary glands from 21-day PPZ-treated rats

exhibited a level of lobuloalveolar development that was similar to the mammary glands from 12-day pregnant animals (Fig. 1B, panels ii and iii, respectively) however with less side-branching than the glands from 12 day pregnant animals. In addition, 21-day E+P stimulated rats expressed β-casein at a level that was readily detectable by Northern analysis using total RNA (data not shown). In contrast, mammary glands from AMV control animals, receiving blank pellets showed no significant alveolar differentiation, suggesting that alveolar development in the E+P-treated and PPZ-treated groups was a result of hormonal stimulation. Previous studies demonstrated that treatment with either E+P or PPZ confers a high level of proliferation and lobuloalveolar differentiation to the mammary glands of treated animals (22). However hormonal regulation has not been examined beyond the gross morphological level to determine if the level of development is the same in both cases.

At the end of the 21 day-treatment period, hormonal stimuli were withdrawn and remaining animals allowed to undergo involution for 28 days. This resting phase mimics the period of involution that occurs after a normal pregnancy and lactation and represents a period of extensive tissue remodeling. At the end of involution, the gland generally reaches a quiescent state, resembling that of the mature virgin animal (2). Figure 1C shows whole mount analysis of 28 day involuted mammary glands from 95 day old animals following prior treatment with either E+P (panel i) or PPZ (panel iii) and their age-matched controls (panels ii and iv, respectively). As reported in previous studies (11), similar morphological characteristics were observed in all of the treatment groups, indicating that there were no persistent structural differences as a consequence of hormonal stimulation of the gland.

Differentially expressed genes identified by SSH

To help elucidate the mechanisms governing this hormone-dependent parity-related phenomenon, we initially screened for molecular markers of an altered cell population, or changes in the subset of genes that were persistently expressed as a result of prior hormonal stimulation. Using the experimental paradigm described above, poly A+ RNA was isolated from pooled total RNA samples corresponding to either E+P treated or AMV control glands following a 28-day involution period. SSH was then employed to generate an E+P subtracted library (as described in Materials and Methods). The library was screened by differential reverse Northern blot analysis (26), to identify genes that are differentially induced by this hormone treatment. An example of the screening method is presented in Fig. 2, illustrating the utility of this approach for identifying differentially—expressed markers by a difference in hybridization signal intensity on duplicate filters. Fig. 2A shows one set of 96 clones, amplified by PCR, arrayed by high-density gel electrophoresis and photographed to ensure even loading between duplicates. Fig. 2 B and 2 C show duplicate membranes, each containing DNA corresponding to the complete set of markers shown in Fig. 2A. Duplicate membranes were screened by reverse Northern analysis, using probes corresponding to either the E+P subtracted (Fig. 2B) or virgin subtracted (Fig. 2 C) amplified cDNA used in the construction of the SSH library (as described in the Materials and Methods). This procedure resulted in the identification of several markers that were characterized in greater detail in Fig. 3. These markers are indicated by boxes and include both low abundance genes, such as RbAp46, Prl-1, PP1δ and hnRNP A1 (Fig 2 B and C; i, ii, iii, v, respectively) as well as more highly expressed markers such as transferrin and α-casein (Fig 2B and B; iv and vi, respectively).

Despite the morphological similarity of the tissues used in this procedure, 24% of the 864

randomly selected amplified markers appeared to be differentially expressed, based on the results of the high density differential reverse Northern blot analysis (Table I). Selected differentially expressed clones (n=203) were sequenced, and their identity determined using the BLAST homology search algorithms and the public sequence databases available through NCBI. Clones were classified as known, homologous, unknown or novel depending on the stringency of the search criteria and the databases from which the sequence matches were found. A summary of this analysis is presented in Table I. Thus, according to these criteria detailed in the footnote to Table I, 61.5% of the markers sequenced corresponded to known genes, 10.8 % to homologous genes and 27.5 % to genes of unknown function.

A summary of fifty known genes identified using this strategy, is presented in Table II. These genes fall into several distinct categories: those involved in normal metabolism and homeostasis of the gland (metabolic enzymes, transport molecules); those involved in cell-cell contact and extracellular matrix; and regulatory factors (signaling molecules, transcription factors, etc.). A number of these genes (such as RbAp46, Nap1, CDC42) encode molecules of known relevance to cellular proliferation pathways (27-29). Surprisingly, several well-characterized markers of mammary differentiation (such as the milk protein genes, transferrin, α-casein and κ-casein) were also identified using this screen, and displayed persistent expression in E+P treated glands. These markers were highly represented in the subset of clones that were selected for sequencing and accounted, respectively, for 18.2%, 3.9 % and 2.9% of the total number of clones sequenced. These observations suggest that there is some degree of persistent differentiation in the mammary epithelium following parity (or treatment with E+P). However, whether this differentiation persists as a consequence of direct effects on the mammary gland or is a consequence of an altered hormonal axis in the E+P-treated animal has not yet been

determined. Similar findings, involving sustained expression of these markers have also been reported from studies in the parous mouse (3).

Another marker that was highly represented was the mRNA for the stearyl-CoA desaturase, an enzyme that catalyzes the rate-limiting step in the biosynthesis of monounsaturated fatty acids (30). Stearyl-CoA desaturase is expressed in an isoform- and temporally-specific manner during development, depending on the tissue of origin (31). The fact that it is so highly represented amongst the subset of clones sequenced suggests that there are also persistent developmental changes in the mammary stroma in response to E+P however, the implications of this observation to the gland as a whole, have also not yet been determined.

SSH has been used in a variety of experimental contexts for the identification of differentially expressed genes. The efficiency of the subtraction procedure is validated by the fact that several markers isolated using this approach (such as α-casein) have been isolated in an independent study, using quite different approaches to identify genes that are differentially expressed in response to parity. In addition, parallel studies using SSH to identify markers that are down-regulated by E+P have isolated quite a different set of markers from those described in the present study (M. Ginger and J. Rosen, unpublished).

Validation by Northern analysis

In order to validate the SSH screen and reverse Northerns (described above) a selected subset of markers were characterized further by Northern blot analysis to confirm their pattern of expression in the E+P pretreated, involuted gland compared with the AMV control tissue. A pooled RNA fraction was used in these analyses to control for the effects of variation in estrus cycle within an age-matched cohort of animals. Northern blots were hybridized with

radiolabelled probes prepared from the partial cDNA fragments isolated from the SSH library. The expression of individual markers was determined by quantitative analysis of the subsequent hybridization signals, using cyclophilin as a normalizing control. Twenty-one markers were examined in this manner — eighteen of which were confirmed as differentially expressed, based on quantitative Northern analysis of their expression in the E+P-treated gland vs. AMV. Since the SSH strategy utilizes an amplification step to permit the detection of low abundance markers, it is possible that some sequences will be preferentially amplified, resulting in false positives. The observation that 85 % of the markers examined by Northern analysis were true positives suggests that the screening criteria described above, resulted in the stringent selection of differentially-expressed markers.

The results of the Northern analysis are summarized in Table III, and highlight several genes that might be putative candidates involved in the molecular pathways that are targets for estrogen /progesterone mediated changes associated with parity-related protection of the gland. Representative Northern blots of markers demonstrating quantitative changes in E+P-treated glands are shown in Figure 3. Several interesting observations, accounting for the differential gene expression were revealed by these Northern analyses. Some of these genes were expressed as a single transcript and showed a small quantitative change, e.g. α-casein (Fig. 3a 1.8-fold difference), Nap 1 (Fig 3c, 8.4-fold change) cdc42 (Fig. 3d, 2.1-fold change), RbAp46 (Fig 3q, 3.5-fold change). Others were detected as multiple transcripts and one or more of these transcripts were differentially expressed, e.g. follistatin-related protein (Fig 3g, 2.1-2.3-fold change), D.E10, (Fig. 3h, 2.9-3.4 fold change), hnRNP A1 (Fig. 3 k, 3.8-fold change). In the third case, several transcripts were observed and all of the transcripts appeared to be differentially expressed, e.g. G.B7 (Fig 3l, 5-7.2-fold change). Whether these transcripts arise as

differently spliced forms of the same gene product, or closely related members of a gene family remains to be determined. Furthermore, the abundance of these transcripts identified by SSH and reverse Northern blots varied considerably from highly abundant mRNAs, such as those encoding α-casein, easily detected following only a few hour exposure (Fig. 3a), to low abundance mRNAs, such as RbAp46, which required more than a week of exposure (Fig.3q). On the basis of this preliminary screen of markers up-regulated by treatment with E+P, two genes were selected for further characterization in greater detail: RbAp46 and a novel clone (designated G.B7).

Cloning of full-length GB7

Clone G.B7 corresponds to a 764 bp fragment, isolated in the SSH screen described above. Analysis of the 764 n.t. of sequence available for this cDNA revealed no significant homology with any sequences in the publicly accessible nucleotide databases — hence it represented a potentially novel gene product. To further elucidate the function of this cDNA, we constructed and screened an E+P-treated mammary gland cDNA library and isolated several full-length clones corresponding to the different sized transcripts observed by Northern analysis (shown in Fig. 3l). Three of these clones (6.3, 2.4 and 2.2 kb in size) were sequenced in their entirety. Analysis of the resultant sequences suggested that the different-sized transcripts observed by Northern blot analysis arose as a result of differential splicing of a single gene product. The full-length sequence of the largest of these clones (6.3 kb in length) was deposited in Genbank, with the accession # AY035343.

In an attempt to determine the function of this gene, we submitted the full-length, 6.3 kb sequence to database searching using BLAST and TBLASTN search algorithms (with six-frame

translation and BEAUTY post-processing). The results of this analysis did not reveal significant homology to any known gene or protein motif. However, we found that a short section of the 5 and 3 region of this gene (n.t. 1125-1490 and 5920-6203 of the full-length sequence) exhibited homology with several rat and mouse EST clones. The best matches were to accession #s BG079981, BE119249 and BG079981 and revealed an identity of 85-97 % to these regions of ~300 bp in the full-length G.B7 sequence. In addition, we performed homology searching using the recently assembled human genome sequence database and found four regions of homology to human chromosome 2. These homologous regions corresponded to n.t. 2334-2419, 2435-2601, 2745-2789 and 5157-5437 of the full-length G.B7 sequence, with identities of 93, 83, 95 and 80 % respectively ($E < 1^{-9}$). This sequence maps to region 2q33 of the human genome and spans a known chromosomal break-point, which is associated with a number of human cancers, including breast adenocarcinoma (32). No open reading frames (ORF) or ESTs have been identified in the human sequence encompassing the region of homology with G.B7. However this may be a consequence of incomplete annotation of genome database or the failure of the search paradigms to detect a non-translated RNA. Indeed, we have been unable to detect any significant ORFs longer than ~200 n.t. in the full-length G.B7 cDNA. *In vitro* translation experiments, with appropriate positive controls run in parallel to validate the assay, also failed to detect any translation product for either the 6.3 kb, 2.4 kb or 2.2 kb forms of this RNA (data not shown).

Expression of GB7

Northern analysis demonstrated a 4- to 8-fold induction (depending on transcript) of GB7 transcripts in the glands of hormone-pretreated rats compared to those in AMV virgin controls.

Multiple tissue Northern analysis suggested that this gene is also expressed in the testes, liver and heart of rats (data not shown), but its hormonal regulation in these tissues has not been determined. Since the mammary gland contains a heterogeneous population of cells (33), including fibroblasts, adipocytes and several types of epithelial cells, it was important to determine in which cell types this RNA transcript was expressed. To investigate the spatial pattern of G.B7 expression in response to treatment with E+P and PPZ, we employed *in situ* hybridization analysis using a ^{33}P-labeled probe generated from the 764 bp fragment isolated from the SSH library. As shown in Fig 4 a, G.B7 mRNA was highly expressed in the epithelium, but not the stroma of 12-day pregnant mammary glands. Furthermore, its expression appeared to be localized to the differentiated lobuloalveoli. In addition, G.B7 was persistently expressed in residual alveoli of the regressed gland following E+P-treatment and 28 days of involution (Fig. 4c). By comparison, only a very faint hybridization signal was detected in the PPZ-treated gland, following 28 days of involution (Fig. 4e). No signal was detected in the 95-day old AMV gland (Fig. 4 g) or with the sense control (data not shown). Interestingly, the expression of G.B7 in the E+P-treated and 12-day pregnant animal is primarily confined to the lobuloalveolar structures of the gland, and not in the ducts. Furthermore, persistent expression of G.B7 requires prior exposure to both E and P, as either E or P alone did not induce expression (data not shown). This pattern of expression suggests that it represents a potentially interesting and biologically relevant marker of parity-related protection.

Expression of RbAp46

Northern analysis demonstrated that RbAp46 is persistently up-regulated in mammary glands of E+P treated rats, compared to AMV controls. To investigate the spatial pattern of its expression,

we again employed *in situ* hybridization analysis (Fig. 5). This analysis showed that RbAp46 was expressed at a high level in the lobuloalveoli at mid-pregnancy (Fig. 5, panel a and c). In addition, RbAp46 was persistently expressed in the alveolar structures of the E+P-treated gland following 28 days of involution (Fig. 5, panel d). By comparison, RbAp46 expression was barely detectable in the PPZ-treated, 28-day involuted gland (Fig 5, panel e) or the 95-day old AMV control gland (Fig. 5, panel f). These results indicate that prior exposure to both E and P is required for the maximal persistent expression of RbAp46.

Discussion

The E+P-treated rat is a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. Two prevailing models have been proposed to account for this phenomenon: In the first model proposed by Russo and Russo (34), the refractory state results from terminal differentiation of the mammary epithelia during pregnancy. According to this hypothesis, terminal differentiation rids the gland of a specific population of susceptible cells (present in the terminal end bud and terminal ducts) that are considered targets for tumorigenesis. Protection is manifested as a change in cellular kinetics, an alteration of the properties associated with carcinogen uptake, binding and metabolism and an enhanced capacity for DNA repair (14, 25, 34-36).

According to the second model, the refractory state is intrinsic to the host, and is mediated by changes in the levels of levels of systemic hormones (such as growth hormone and prolactin), which may subsequently down-regulate the expression of receptors for hormones and growth factors (21). These models may not be mutually exclusive, and while both attractive, neither has

gained sufficient mechanistic evidence to confirm their validity. In addition, comparison of these hypotheses is hampered by the fact that while one employs a pre-treatment model of hormonal protection, the other utilizes a post-treatment paradigm. In the first case, differentiation is proposed as the mechanism for the protection, while in the post-treatment model, an agent that causes morphological differentiation of the gland does not confer refractoriness against cancer (22). Furthermore, the differentiation hypothesis is inconsistent with the observations that the cellular kinetics and morphological characteristics of the AMV and involuted gland are virtually identical (11).

To overcome the limitations of the aforementioned models we, and others (11), have proposed an alternative hypothesis, i.e. that the normal hormonal milieu that is present during pregnancy results in persistent changes in the molecular pathways governing cell fate in a defined population of cells in the mammary gland. In this respect, the elucidation of novel targets for estrogen and progesterone action in the mammary gland, is crucial to our understanding of how an aspect of normal development might mediate the response of the organ to future proliferative signals. We have selected the 28-day involuted gland as the model for the molecular studies presented above, because it is morphologically similar to that of mature virgin animals of the same age. Thus, we are provided with an appropriate control to examine molecular differences in two tissues that are very similar morphologically.

In the expression studies presented in Fig. 4 and 5, we used PPZ treatment to distinguish between the molecular pathways induced by either E and P, or P alone. PPZ is a dopamine receptor agonist that alters the hormonal axis to the extent that both serum prolactin and progesterone levels are elevated but estrogen is not significantly increased. As stated above, PPZ caused differentiation of the gland, but not protection, in a post-treatment model (22). Whether

it confers protection in the pre-treatment paradigm described herein, remains to be tested, but in previous studies performed in the Wistar-Furth rat model both E and P were required for subsequent protection from carcinogenesis (37). The fact that we have observed slight morphological differences between the PPZ- and E+P-treated gland (following 21 days of stimulation) suggests that different molecular pathways are invoked by E+P-mediated or PPZ-mediated development of the gland.

Suppression subtractive hybridization has been used in a variety of experimental contexts for the identification of differentially expressed genes. It has certain advantages over many conventional methods of gene discovery in that it is capable of identifying both known and novel as well as low abundance genes. The last point is of particular interest, because alternative methods, such as microarray analysis often fail to detect quantitative changes in low abundance transcripts that are detected by more sensitive techniques such as SSH (38). In many cases, it is necessary to use poly A+ Northern analysis to verify the expression of markers identified by SSH. In the present study, we have demonstrated the utility of this approach for isolating markers that are persistently up-regulated by treatment with estrogen and progesterone in the mammary gland. This has highlighted a number of markers of prospective importance to E- and P-dependent pathways in the mammary gland, potentially leading to the determination of cell fate. Whilst some of these markers represent genes of, as yet, unknown function, others encode molecules of known relevance to the pathways involved in cellular proliferation and differentiation. It is difficult to reconcile such a diverse group of markers with the molecular events that might be involved in conferring protection to the gland. However, it is possible that some contribute in an independent, but stochastic fashion to influence the processes controlling cell fate. Consideration of their functions may lead to a greater understanding the significance of

their persistent expression in the E+P-treated gland. For example, carcinogen treatment of the age-matched virgin adult gland leads to a proliferative burst. This proliferative burst is attenuated in the parous and E/P treated gland (11). Several of the genes up-regulated in the E/P treated gland could play a role in this proliferative block.

The marker C.B10 encodes the rat homologue of the human gene for hnRNP A1 (accession # M12156). Heterogeneous nuclear ribonucleoproteins (hnRNPs) are well recognized as regulators of alternative mRNA splicing in the nucleus. Alternative mRNA splicing is often a key mechanism in the process controlling development and cell fate (39). Aberrant mRNA splicing has been associated with the development of breast cancer (40), as have changes in the level of particular splicing factors (41). Thus, it is conceivable that sustained expression of hnRNP A1 may be important for maintaining the appropriate expression of certain regulators of cell growth. In addition, recent evidence suggests that hnRNP A1 may also play a role in the protection of the ends of mammalian telomeres from either degradation or telomerase-dependent extension (42). Since the extension of telomeres is implicated in the immortalization of cells (2), leading to uncontrolled proliferation, the telomerase-mediating properties of hnRNP A1 may also be important for maintaining the refractory state.

Clone D.G2 encodes the rat gene for follistatin-related protein (FRP; accession # U06864). Follistatin-related protein is a member of wider family of follistatin-module containing proteins that have been ascribed the function of modulating the effects of cytokine and growth factor signaling (43). The mouse homologue of rat FRP, TSC-36 was originally isolated as a TGF β-inducible gene in osteogenic cells (44) — suggesting an antiproliferative function for this gene. Subsequent studies have shown that overexpression of TSC-36/FRP can inhibit the proliferative activity of a human lung cancer cell line (43). In addition, TSC-36/FRP

is expressed in a temporally and spatially specific manner during mouse embryogenesis (45). TSC-36/FRP expression is regulated by estrogen in osteoblastic cells (46). Together, these studies imply that FRP may play a role in conferring estrogen-dependent changes during development of the mammary gland.

Two markers encoding phosphatases, Prl-1 (accession # L27843) and the delta subunit of protein phosphatase 1 (PP1δ; accession # D90164) also showed sustained expression following E+P treatment in the rat mammary gland. Both Prl-1 and PP1δ, are members of large superfamily of protein tyrosine phosphatases, which contain a PTP-catalytic domain signature sequence (47). The opposing functions of many kinases and phosphatases play a critical role in the regulation of diverse cellular processes including the modulation of gene expression, cell-cycle progression and intracellular transport, to name a few. It is, therefore, likely that they also participate in various process involving developmental morphogenesis and the determination of cell fate. Prl-1 appears to exhibit dual functions depending on the cellular context — in the liver it is associated with proliferation (48), however in the intestine it is expressed in the differentiated epithelium of the villus, but not in the crypts (49). Prl-1 is also expressed in a temporal and spatially specific manner in the mouse embryo (50). Its role in mammary gland development has not yet been described.

We have characterized two of these markers (G.B7 and RbAp46) in greater detail, and found that both are persistently expressed in a specific population of epithelial cells following treatment with estrogen and progesterone. The first of these markers encodes a gene of as yet unknown function that shows a 5- to7-fold induction in the glands of hormone-treated rats compared to those in AMV controls. The observation that it is homologous to sequences in a region of human chromosome 2, and spans an area that is known as a chromosomal break-point

in a number of human cancers, is tantalizing and merits further investigation. However, we have been unable to identify a putative translation product. Since this gene is clearly expressed as an mRNA transcript (based on the Northern analysis shown above, and the presence of related sequences in the EST database), and was persistently induced following treatment with E+P, we speculate that GB7 may instead function as a non-translated regulatory RNA. There is some precedence for this hypothesis: several studies in a range of organisms (including *C. elegans*, *Drosophila* and mouse) have shown that RNA molecules may function in a regulatory context without themselves being translated. In *C. elegans*, two short non-coding RNA species (*lin-4* and *let-7*) have been identified that repress the function of several genes that mediate developmental control pathways (51). These RNAs appear to exert their repressive effects, by binding to complementary sequences in the 3 -UTR of their mRNA targets, and thus prevent translation. In *Drosophila*, a family of non-coding RNAs, encoded by the roX genes, is involved in chromatin remodeling leading to dosage compensation of the male X chromosome (52). In yet another example, the RNA molecule SRA acts as steroid receptor coactivator in the SRC-1 complex (53). Recent studies suggest that many further examples of RNAs with regulatory functions will be revealed (54).

The Retinoblastoma (Rb)-associated proteins (RbAp46, and the closely related molecule RbAp48) comprise part of a small gene family with homology to the yeast molecule MSI1 (a negative regulator of the Ras-cyclic AMP signaling pathway) (27, 55). Although originally isolated as protein components that bound to Rb by affinity- chromatography (55), preliminary studies suggests that these molecules might play a broader function in the regulation of such processes as cellular proliferation and differentiation. Overexpression of either RbAp46 or RbAp48 can substitute for the activity of MSII in mutant yeast strains. In addition, RbAp46

appears to be a downstream target of the Wilm s tumor suppressor protein (WT1), and overexpression of this gene can suppress the growth of transfected cells in culture (56). More recent studies have shown that these proteins also interact with the breast cancer tumor suppressor protein, BRCA1 (57). Furthermore RbAp46 and RbAp48 interact directly with histones H3 and H4 (58) and are components of multisubunit complexes that are involved in histone deacetylation, histone acetylation, nucleosome disruption and nucleosome assembly (59-61). In this capacity, RbAp46 appears to be involved in both *de novo* acetylation/deacetylation of the nascent chromatin (possibly leading to permanent imprinting of specific gene expression) as well as the targeted repression of gene activation through its association with the Sin3/HDAC and NuRD complexes (60). Together these studies imply a model in which RbAp46 through its actions as an adapter protein, might serve as a means of recruiting these chromosome remodeling activities, leading to persistent changes in gene expression in the glands of parous animals.

Epigenetic changes can thus provide an enduring memory that pre-determines cell-fate and prevents cell-lineage aberrations, leading to cancer (62). This offers a highly plausible explanation for the persistent changes in gene expression that have been observed in the mammary glands of parous animals. It is clear that further studies are necessary to fully elucidate the function of these markers in the parous mammary gland. However, the elucidation of markers that show persistent changes in gene expression in response to exposure to E and P is critical for understanding the molecular pathways that are altered in the parous gland and modulate the response of the gland to further proliferative stimuli. In this study, we have identified a number of such markers that warrant further study. These results provide the first support on the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland and can affect the response of

mammary epithelial cells to carcinogenic insults.

Materials and Methods

Animals

Thirty-five day old virgin and five day-timed pregnant Wistar-Furth (WF) rats were purchased from Harlan Sprague-Dawley, Inc. (Chicago, IL). Animals were housed using approved AALAC guidelines in plastic cages containing wood-chip bedding, under conditions of a twelve hour light-dark cycle and permitted *ad libitum* access to food and water. All experiments were performed in accordance with the NIH guidelines for the care and use of experimental animals.

Hormonal Manipulation

Forty-two day old WF rats were treated with a defined hormonal regimen to mimic the protective effects of an early full-term pregnancy using the experimental paradigm described previously by Sivaraman *et al.* (11). Rats were divided into two groups (n=20 per group); those receiving the hormonal stimulus and those serving as age-matched virgin (AMV) control subjects. In both cases, the rats were treated with a priming dose of estradiol benzoate dissolved in sesame oil (2.5 μg in 0.1 ml; s.c.) to synchronize estrous both within and between the two groups. Three days subsequent to the estradiol boost, animals in the experimental group were treated with E and P by subcutaneous implantation of a pellet containing 20 μg E and 20 mg P in a beeswax medium implanted in the dorsal region of the back. Conditions for the preparation of the E/P pellets have been described previously (11). AMV control animals received blank pellets containing the vehicle alone. Pellets were replaced after 10 days to provide hormonal stimulation for a total of 21 days. The hormonal stimulus was continued for twenty-one days to mimic the period of a full-term pregnancy. After this phase, the beeswax pellets were removed and the rats subjected

to a resting period of 28 days to allow the mammary glands to involute (Figure 1A).

To study the effects of differentiation, in the absence of the hormonal changes conferred by pregnancy or E+P treatment, 45-day old Wistar-Furth rats were treated with perphenazine (PPZ) using a modification of the experimental paradigm presented above. Rats were divided into two groups, ten animals per group; those receiving the hormonal stimulus and those serving as age-matched virgin (AMV) control subjects. Rats in the treatment group then received a s.c. injection of PPZ (Sigma; 5mg/kg in 0.03M HCl) five times per week for a period of three weeks. Controls received the vehicle alone. At the end of the treatment period the rats were again subjected to a resting period of 28 days to allow the mammary glands to involute.

Tissue Collection

Animals were sacrificed by an intracardial injection of 0.05 ml ketamine/ xyalazine/acepromazine. Inguinal # 4 mammary glands were harvested from 12-day pregnant, 21-day E+P treated, 21-day perphenazine-treated, 28-day involuted (E+P, PPZ) and their AMV control subjects. Tissues were either flash frozen in liquid nitrogen for Western and Northern blot analyses or fixed in 4 % paraformaldehde (PFA) in PBS at 4 ßC for 18 h for *in situ* hybridization analysis. For the 12-day pregnant samples, pregnancy was confirmed by dissection of the uterus and by the presence of a normal conceptus. For E+P- and PPZ-treated animals, hormonal stimulation of the gland was routinely confirmed by removing the left # 4 mammary gland immediately following the 21 day treatment period or following a 28 day involution period, and subjecting it to whole mount analysis as described previously. In this case, mammary glands were fixed in 10 % neutral buffered formalin for 24 h and stained as described previously (63). Stained glands were examined to ensure that they displayed morphological

development consistent with the particular regimen.

RNA Isolation

Total RNA was isolated from frozen tissues by homogenization in RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RNA fractions from different animals from the same group were combined to minimize variation between individuals and the poly A fraction isolated from this pooled RNA sample using the PolyATtract¤ mRNA purification system (Promega, Madison, WI). RNA quality and yield was determined by spectrophotometric measurement and the RNA stored at — 70℃ until used.

Suppression Subtractive Hybridization

SSH was performed using the PCR-Select" cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA) in accordance with the manufacturer's instructions, but with the following modifications. Poly A+ RNA was isolated from pooled total RNA samples (n=18 each) corresponding to either E+P treated or AMV control glands following a 28-day involution period. Complementary DNA fractions were synthesized from 2 μg of poly A RNA from each tissue pool and then the AMV cDNA used as a driver to subtract molecules common to both populations of RNA from the E+P tester cDNA. The efficiency of the subtraction procedure was monitored by gel analysis of the amplified products before and after subtraction and by depletion of G3PDH in the subtracted versus the non-subtracted cDNA. Subtracted products were subjected to PCR-based amplification in which the primary and secondary PCR conditions were altered as follows to optimize product formation: in the primary PCR, the annealing temperature was reduced to 64 °C and the number of cycles raised to 30. In the secondary PCR,

a final extension cycle of 7 min was added, and the amplified products were A-tailed by incubation at 72 °C for 15 min in the presence of 0.5 U Taq polymerase (Gibco BRL). The resultant subtracted amplified cDNA products were purified and cloned into a pGEM¤ -T Easy TA cloning vector (Promega, Madison, WI). The ensuing E+P SSH library was propagated by transformation into Epicurian Coli¤ XL2-Blue ultracompetent cells (Stratagene, La Jolla, CA) according to the recommendations of the supplier. Recombinant E+P SSH clones were selected by plating onto 150 mm diameter plates supplemented with isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-gal).

Differential Screening of the E+P SSH Library

Colonies (n=864) from the E+P SSH library were selected at random and inoculated into 100 µl of LB supplemented with ampicillin (100 µg/ml) in 96-well round-bottomed microtitre plates. Bacteria were incubated at 37 °C overnight on a shaking platform and then 3 µl of the subsequent bacterial culture transferred to individual wells of a 96-well PCR plate (Perkin-Elmer). Bacteria were lysed by heating to 95 °C for 5 min and the recombinant DNA inserts amplified using nested primers complimentary to the adapter fragments used in the library construction, as described in the PCR-Select" cDNA Subtraction Kit manual. PCR reactions typically contained 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.3 µM of each primer and 1 U Taq DNA polymerase (Gibco) in a total volume of 20 µl in the presence 1X standard PCR buffer. Taq polymerase was preincubated with Taqstart antibody (Clontech) for 30 min at RT before addition to the reaction mix. Amplification was performed using 23 cycles each of 94 °C for 30 s and 68 °C for 3 min and 30 s. Equal volumes of the amplified products were arrayed, in duplicate, on high-density format 1.5 % agarose gels (Centipede" gel electrophoresis chambers; Owl Scientific, Woburn,

MA), as described by von Stein *et al.* (26) and then transferred onto charged nylon membranes (Hybond N+, Amersham) using standard protocols.

The resulting duplicate filters were screened with double stranded cDNA probes corresponding to either reverse or forward subtracted cDNA prepared during the library construction process. Hybridizations were performed under stringent conditions as described in the PCR-Select Differential Screening Kit Users Manual (CLONTECH laboratories, Inc.). Hybridization signals were visualized by exposing the hybridized filters to BioMAX MR film in the presence of a BioMAX MS intensifying screen (Eastman Kodak Co.) and the signals of replicate clones compared. Clones displaying a differential pattern of expression were selected for further analysis and plasmid DNA isolated using a Quantum miniprep kit (BioRad).

Isolation of full-length GB7

A rat E+P cDNA library was prepared from poly A+ RNA isolated from 28 day involuted E+P- treated mammary glands and ligated into a lambda ZAP Express bacteriophage vector (Stratagene) in accordance with the manufacturer s instructions. A total of 5×10^5 recombinant plaques from the resulting amplified library were screened by standard protocols, using a random primed [α-^{32}P]dCTP-labeled cDNA probe corresponding to the 764 bp GB7 fragment isolated from the E+P SSH library.

DNA sequencing and analysis

Purified plasmid clones were subjected to di-deoxy sequencing using an ABI 377 automated sequencer (PE Applied Biosystems) at the DNA Sequencing Facility of the Child Health Research Center, Baylor College of Medicine. Clones from the SSH library were sequenced

using the T7 universal primer. Full-length GB7 clones were sequenced using sequence-specific internal oligonucleotide primers obtained from IDT (Integrated DNA Technologies, Coralville, IA.). The resulting sequence data were analyzed using the homology analysis software, BLAST and BEAUTY, available respectively through the National Center for Biotechnology Information (NIH, Bethesda, MA.) and the BCM Search Launcher (Human Genome Sequencing Center, Baylor College of Medicine).

Northern analysis

The polyA+ RNA fraction from E+P-treated 28 day involuted and AMV control mammary glands was prepared from a pooled total RNA sample as described above. Poly A+ RNA (2μg/lane) was resolved by electrophoresis on 1.2 % agarose/0.66M formaldehyde gel and subsequently transferred onto a charged nylon membrane. The blots were hybridized with [α^{32}P]dATP-labeled cDNA probes prepared from selected clones isolated from the E+P/SSH library, stripped and re-probed with a cyclophilin probe. Hybridization signals were detected by exposing the filters to BioMAX MR film in the presence of intensifying screens. Several exposure times were used to ensure that the signal from individual hybridizations were in the linear range for the film. Films were scanned using densitometry (Molecular Dynamics, Inc., Sunnyvale, CA.) and quantitation performed using Image Quant 1.1 software (Molecular Dynamics, Inc.). The fold induction of individual markers was determined by normalizing the quantitative data to that obtained from the cyclophilin probe.

In situ hybridization

Paraffin-embedded sections (7 μm) from PFA-fixed tissue were cut and mounted onto Probe-On

Plus charged slides (Fisher Scientific, Pittsburgh, PA.). Sections were deparaffinzed, rehydrated, treated with proteinase K (20 μg/ml) for 15 min, post-fixed in 4 % PFA and prehybridized for 1 h in hybridization buffer [50 % formamide, 5 X SSC, 10 % dextran sulfate, 5 X Denhardts, 2 % SDS, 100 μg/ml denatured salmon sperm DNA] at 38 °C. [α^{33}P]UTP-labeled riboprobes for GB7 and RbAp46 were transcribed, respectively, from 764 and 429 bp fragments isolated from the E+P/SSH library cloned into pGEM® -T Easy. The RbAp46 fragment corresponded to n.t. 1279-1708 of the full-length rat RbAp46 cDNA (Genbank Accession No. AF090306). Hybridization was performed at 42 °C overnight in the presence of 1 X 10^5 cpm/μl radiolabeled cRNA probe. Coverslips were removed in the presence of 4 X SSC (55 °C), sections were washed in 2 X SSC / 50% β-mercaptoethanol for 20 min at RT, then digested with RNAse A (40 mg/ml in 2 X SSC) for 15 min at 37 °C. Stringency washes were performed in 0.1 X SSC for 15 min at 42 °C and 0.1 X SSC for 15 min at RT. Sections were exposed to emulsion (Kodak) for 3-5 days and then mounted in Vectashield + 4 , 6-diamidino-2-phenylidole (DAPI) medium (Vector Laboratories, Inc., Burlingame, CA.).

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Figure Legends

Fig. 1. Mammary Gland Morphology Following Different Hormonal Paradigms

A. Experimental paradigm for studying the protective effect of E and P treatment. Forty-two day old Wistar-Furth rats were administered estradiol benzoate (E_2B) by s.c. injection and then a subcutaneous pellet of E+P three days later. The pellets were replaced after ten days to provide hormone treatment for a total of 21 days duration. AMV virgin animals are given blank pellets. After 21 days the pellet was removed and the mammary gland allowed to return to a resting stage, approximating a 28 day cycle of involution. At the end of the 28 day involution period, mammary glands are removed for investigation. **B**. Morphological analysis following hormonal stimulation. Whole mount preparations of inguinal # 4 mammary glands of 66-day old rats, following 21 days of treatment with E+P (i) or PPZ (ii). A mammary gland from a 12 day pregnant animal was used as a positive control (iii), to assess to level of morphological development achieved by the hormonal regimen. A 66-day old AMV animal is shown as a negative control, for mammary development in the absence of exogenous hormones (iv). Arrows indicate morphological differences between the E+P and PPZ-treatments. **C**. Morphological analysis of mammary glands following hormonal stimulation and involution. Whole mount preparations of # 4 mammary glands from 95-day old animals treated with either E+P (i) or PPZ (iii) for 21 days and following a 28 day cycle of involution. AMV animals received either a blank pellet, containing no hormones (ii), or the vehicle alone (as a control for the PPZ treatment; iv).

Fig. 2. Differential screening of the E+P SSH library.

An illustration of the screening procedure used to select markers that are up-regulated in response to prior exposure to E+P. Amplified inserts from 96 randomly selected clones were arrayed, in duplicate, using high-density gel electrophoresis (**A**). Gels were stained with ethidium bromide, to ensure equal loading and then transferred onto charged nylon membrane. Duplicate filters were screened using differential reverse Northern analysis, in which equal specific activity [^{32}P]dATP-labelled probes, generated from either the E+P subtracted (virgin driver) (**B**) or virgin subtracted (E+P driver) (**C**) cDNA were hybridized under stringent conditions. The resulting signal was detected by exposuring the filters to Biomax MR film for 6 h to 2 days. Six differentially-expressed markers, identified using this method, are highlighted by boxes: i) RbAp46; ii) Prl-1; iii) PP1δ; iv) transferrin; v) hnRNP A1; vi) α-casein . The relative position of these cDNAs, in the absence of a hybridization signal is indicated in Fig. 2C.

Fig. 3. Northern validation of the differential screening method.

Selected clones, identified by the SSH library screen (described in Figure 2) were used in Northern blot analysis, to verify differential expression in response to prior exposure to E+P. Northern analysis was performed with 2 μg of poly A+ RNA from either E+P-treated mammary gland or AMV following 28 days of involution. Blots were hybridized with cDNA inserts prepared from the following SSH clones: (**a**) α-casein, (**b**) protein phosphatase 1, subunit d, (**c**) Nck-associated protein 1, (**d**) CDC42, (**e**) clone BB5 which resembles an EST, (**f**) transferrin, (**g**) follistatin-related protein, (**h**) clone D.E10, which resembles an EST, (**i**) clone D.F9 which resembles an EST, (**j**) decorin, (**k**) hnRNP A1, (l) G.B7, (m) protein phosphatase, Prl-1, (**n**) stearyl-CoA desaturase, (**o**) Ring zinc finger protein, (**p**) heme-binding protein, (**q**) RbAp46. Exposure times were as follows 4h (a and f), 12 h (j), 18 h (n), 2 days (k and p), 3 days (o), 4

days (c,,d,and g), 5 days (e,q), 7-8 days (b, h, i, l,, m). Cyclophilin was used as a normalizing control to ensure even loading (**r**). Differentially-expressed transcripts are indicated by arrows.

Fig. 4. A novel marker, G.B7 is expressed in the lobuloalveolar structures of the rat mammary gland in response to estrogen and progesterone.

In situ hybridization analysis showing the localization of G.B7 mRNA in the lobuloalveoli of mammary glands from mid-pregnancy (12 d P) (**a**) and residual alveolar structures of E+P-treated rats (**c**) following 28 days of involution. As a comparison, expression is barely detectable following treatment with PPZ and 28 days of involution (**e**), and in the 95 d AMV glands (**g**). Hybridization signals were captured in the dark field. DAPI-staining of the same field is shown in parallel (**b**, **d**, **f**, **h**). No hybridization was observed with the sense control (not shown).

Fig. 5. Persistent expression of RbAp46 requires prior exposure to both E and P. *In situ* hybridization analysis, showing the localization of RbAp46 mRNA in the mammary gland at 12 d of pregnancy (**a** and **c**) and in E+P-treated (**d**) and PPZ-treated rats (**e**) and AMV (**f**) following 28 days of involution; a, d, e, f, 4 x magnification, c, 10 x. No hybridization was observed with the sense control (**b**).

Table 1. Summary of rat E+P SSH Library Screening

A. Summary of screening strategy	
Screening step	Number of clones examined
Screened by PCR and reverse northern	874
Identification of differentially expressed markers[a]	213
Sequencing	203
Confirm expression by Northern blot analysis	21
B. Representation of cDNA clones sequenced	
Category	% of total clones sequenced
Known genes[1]	61.5
- Transferrin	18.2
- α-casein	3.9
- κ-casein	2.9
- Stearyl-CoA desaturase	2.9
Homologous genes[2]	10.8
Unknown functon[3]	27.5
- ESTs	23.6
- Novel genes[4]	3.9

1. Clones classified as known genes exhibited greater than or equal to 98 % identity with the corresponding database entry.
2. Clones corresponding to known genes for which the identity was not complete (because the relevant rat sequence was not available) were classified as homologous if they demonstrated an expected probably of $< 1^{-5}$.
3. Clones of unknown function were classified as such if they matched sequences in the EST databases, but their function was otherwise unknown.
4. This subset also included a number of novel genes that displayed no significant homology to any sequences in the publicly available databases following extensive analysis using the BLAST, TBLASTN and BEAUTY algorithms.

Table II. Summary of known genes isolated using SSH.

Function	Marker	Accession Number Known	Homologous
Metabolic	Stearyl CoA desaturase	J02585.1	
	Cysteine dioxygenase	D83481	
	GTP-specifice succinyl CoA synthetase		AF058956
	2,4 Dienoyl CoA reductase	D00569	
Nucleic Acid metabolism	5' Nucleotidase	J05214	
	Guanine deaminase	AF026472.2	
Milk protein	α–Casein	J00710.1	
	κ-Casein	K02598	
	β-Casein	NM_017120	
Mineral transport/ metabolism	Transferrin	D38380	
	Heme binding protein	D30035	
	Ceruloplasmin	L33869	
Protein synthesis	Ribosomal protein L18	NM_031102.1	
	Ribosomal protein S20	X51537.1	
	40 kDa Ribosomal protein	D25224	
	Eukaryotic protein translation initiation factor-5	NM_020075.1	
Protein transport	Sec61		U11027
Transcription factor	Zinc finger protein, Pzf		U05343.1
	Ring zinc finger protein, Rzf		AF037205.1
	Zinc finger protein		U90919
	Sp3		AF062567.1
	SHARP-2	AF009330	
Chromatin remodeling	RbAp46	AF090306	
RNA splicing	hnRNP A1	M12156	
	Arginc/serine-rich splicing factor 11		XM_001835.1
Cell cycle control	CDC42		NM_009861.1
	Retinoblastoma	D25233.1	
Signal transduction	Krit-1		AF310134.1
	Nap-1	D84346	
	Collibistin I	NM_023957	
Translational repressor	NAT-1		U76112
Kinase	JAK1		S63728.1
Phosphatase	Protein phosphatase type 1 delta	D90164	
	Tyrosine phosphatase Prl-1	L27843	
	Protein tyrosine phosphatase-like protein, PTPLB		AF169286
Extracellular matrix	Decorin	Z12298.1	
	Follistatin-related protein	U06864	
	Heparin sulfate proteoglycan core protein, Sdc2	NM_013082.1	
	Neuralin-1	AF305714	
Modulation cell growth	Mast cell growth factor		U44725
	EGF-like protein, S1-5/T16	D89730.1	
Cell Adhesion	Podocalyxin	AF109393.1	
	Neural cell adhesion		AF00246
	Cell adhesion molecule		XM_003064.2
Membrane transporter	PMP70, ATP-binding cassette family	NM_012804.1	
	KCl Co-transporter	U55815	
Cell-surface marker	Epithelial glycoprotein antigen, EGP-314	AJ001044.1	
	Interferon gamma receptor	U68272	
Apoptosis	E1B 19K/Bcl-2-binding protein, Nip3	AF243515.1	
Protease	YME1-like ATP-dependent metalloprotease		NM_013771.1

Table III. Genes up-regulated by E+P

Marker	Identity	Accession number Known	Homologous[1]	Transcript[2] size (kb)	Fold difference[3]
A.F10	Zinc fiunger protein, Pzf		U05343.1		-
B.A6	Transferrin	D38380		2.3	2.0
B.B5	EST		AI712974.1	4.2	1.9
				3.7	-
B.B8	Podocalyxin	AF109393.1			1.6
B.D2	Sp3	AF062567.1			-
B.E3	Stearyl CoA desaturase	J02585.1		6.0	4.4
				4.8	-
				3.8	-
B.E4	Decorin	Z12298.1		4.2	1.8
				3.1	1.4
				2.0	-
				0.9	-
B.F2	α-casein	J00710.1		1.3	1.8
B.G10	Zinc finger protein		U90919		-
C.B2	RbAp46	AF090306		1.9	3.5
C.C6	Tyrosine phosphatase Prl-1	L27843		3.2	-
				2.8	6.5
				2.0	4.7
C.F7	Protein phosphatase-1, delta	D90164		2.1	1.4
				1.5	4.3
C.B10	hnRNP A1	M12156		3.2	3.8
				1.8	-
				1.3	-
D.B4	Ring zinc finger protein		AF037205.1	2.2	4.5
D.C6	Nap 1	NM_023957		5.0	8.4
D.F6	Heme binding protein	D30035		1.7	5.3
				1.1	-
D.F9	EST		BF289700.1	2.9	0.27
				2.2	-
				1.6	-
				1.1	2.2
D.G2	Follistatin-related protein	U06864		3	2.3
				2.3	2.1
				1.6	-
				1.3	-
D.E10	EST		BF560064.1	4	3.4
				2.5	2.9
				1.7	-
G.B7	Unknown			6.0	7.2
				4.0	5.6
				2.8	7.2
				2.3	5.0
				1.5	6.1
I.E1	CDC42		NM_009861.1	2.2	2.1

1. For ESTs, the accession # of the closest homologous sequence is shown.
2. Estimated from Northern analysis.
3. Fold difference in E+P vs. AMV – from quantitative analysis of Northerns presented in Fig. 3, using cyclophilin as a normalizing control.

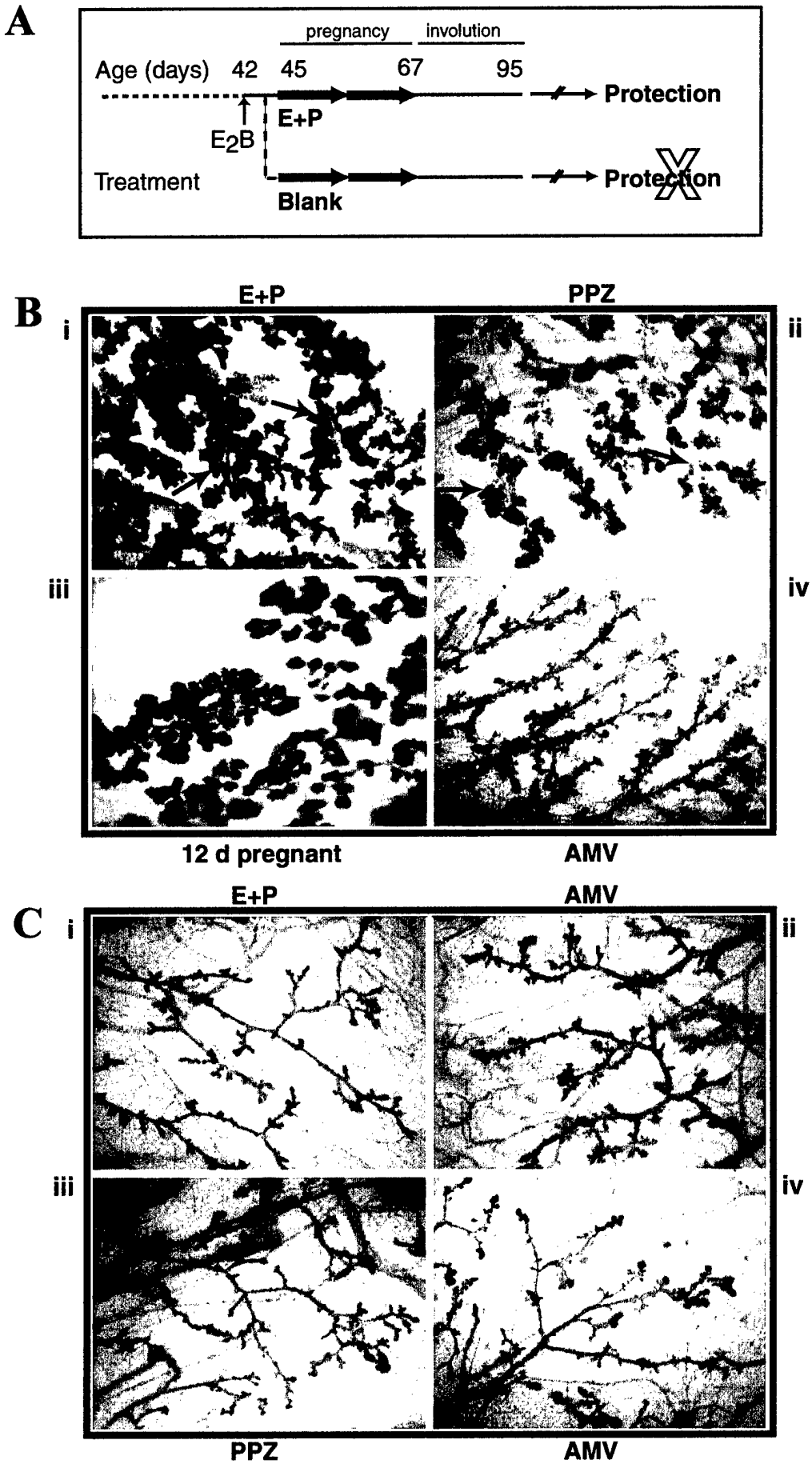
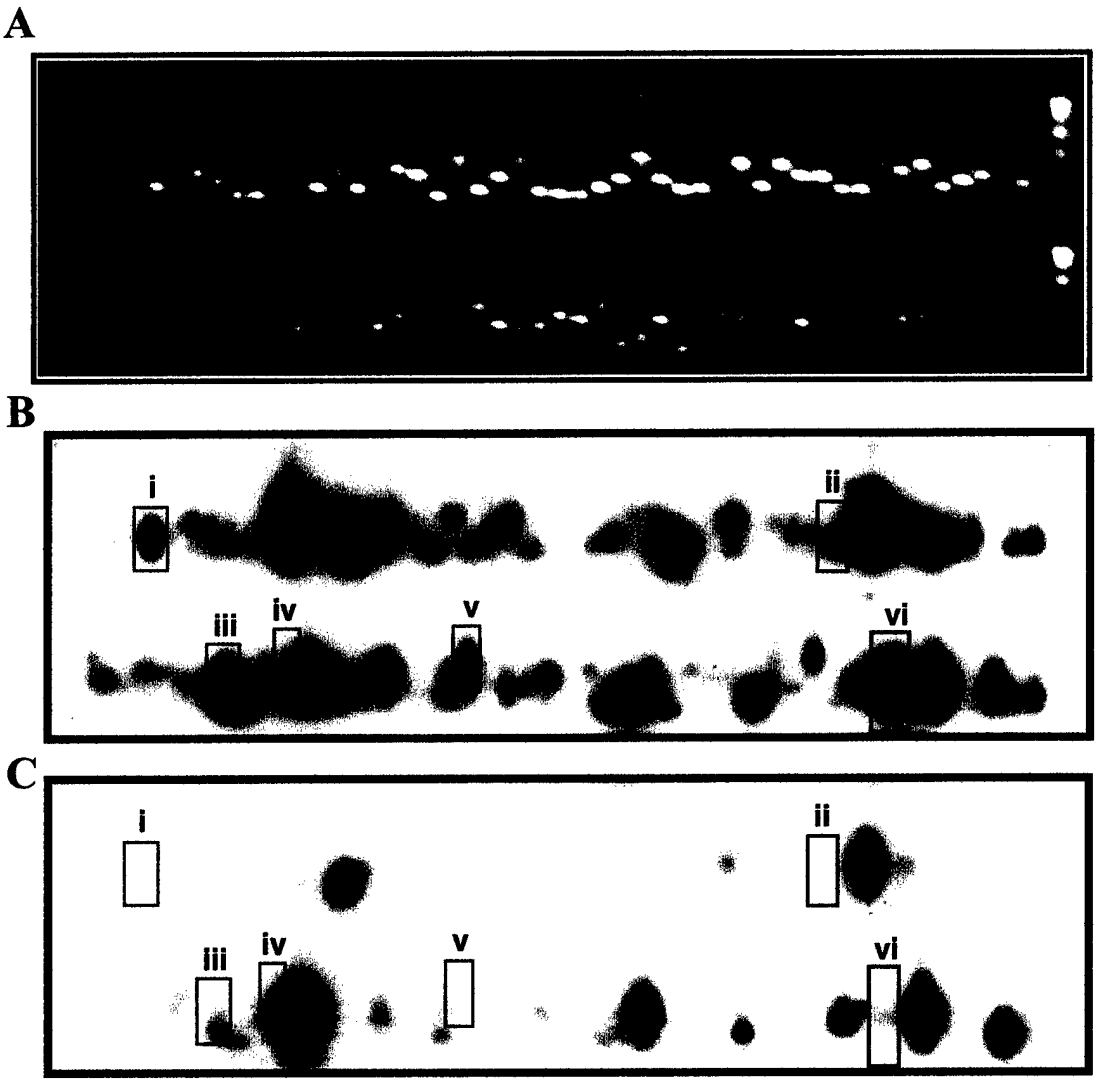

A
pregnancy
involution
Age (days)
42
45
67
95
Protection
E+P
E2B
Treatment
Protection
Blank
B
E+P
PPZ
i
ii
iii
iv
12 d pregnant
AMV
C
E+P
AMV
i
ii
iii
iv
PPZ
AMV

FIGURE 1

FIGURE 2



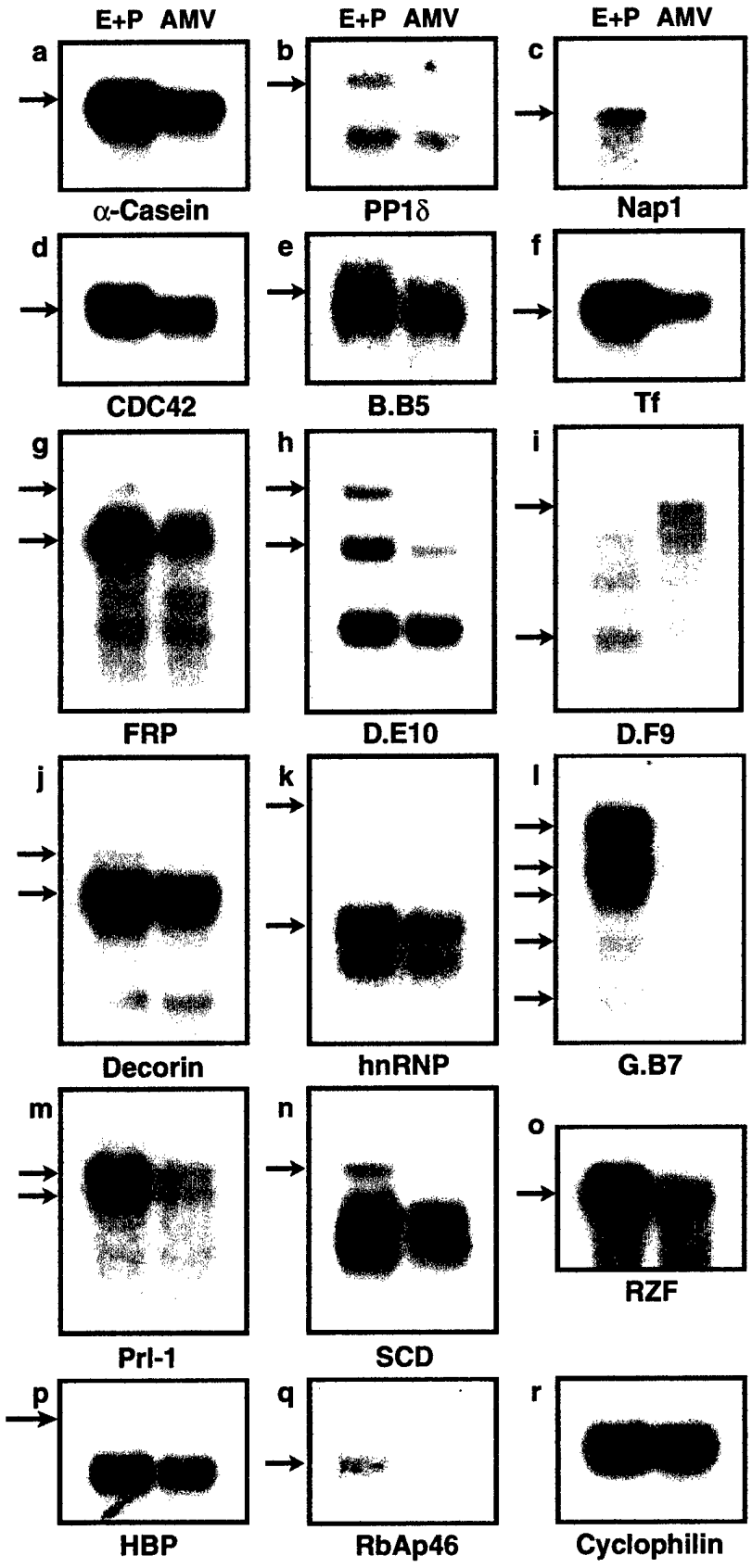
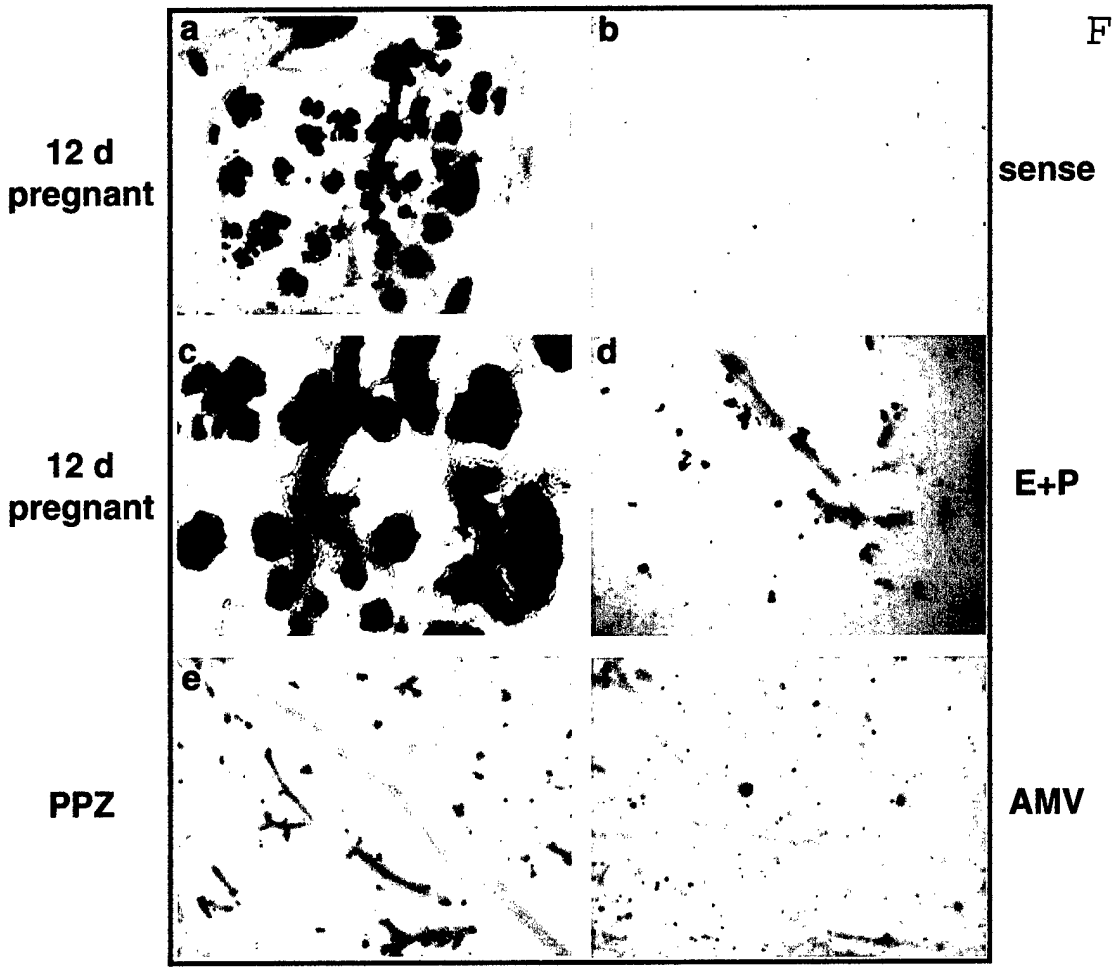

E+P AMV
E+P AMV
E+P AMV
a
b
c
α-Casein
PP1δ
Nap1
d
e
f
CDC42
B.B5
Tf
g
h
i
FRP
D.E10
D.F9
j
k
l
Decorin
hnRNP
G.B7
m
n
o
RZF
Prl-1
SCD
p
q
r
HBP
RbAp46
Cyclophilin

FIGURE 3

FIGURE 4



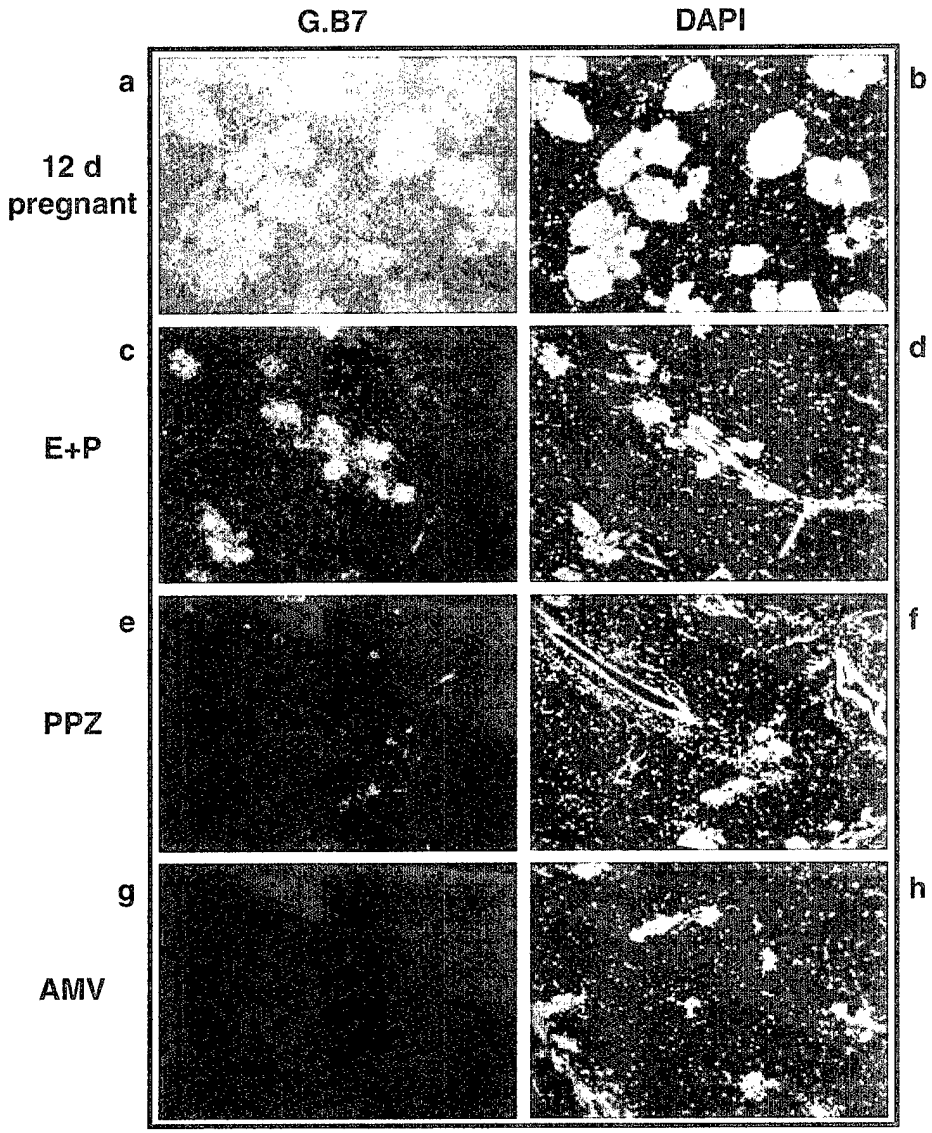


FIGURE 5